Evolving Technology

The tissue-engineered vascular graft using bone marrow without culture

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Objective: To overcome the shortcomings of current vascular grafts, tissue-engineering methods have been applied to cardiovascular regions. We previously reported the creation of a tissue-engineered vascular graft by using vascular mixed cells. However, the cost and manpower for harvesting and culturing the cells was too burdensome. To overcome these drawbacks, we have developed a new method for creating a tissue-engineered vascular graft by using bone marrow cells, which can be obtained easily and used immediately, without cell culture.

Methods: Biodegradable polymers seeded with different types of cells (group V, cultured venous cells; group B, bone marrow cells without culture; and group C, non–cell-seeded graft [as control]) were implanted into the inferior venae cavae of dogs. The grafts were explanted at 4 weeks and assessed histologically and biochemically.

Results: In the histologic examination, a regular layer of Masson-staining collagen fiber and a layer of factor VIII–stained endothelial and anti–α-smooth muscle cell antigen–immunoreactive cells stained in groups V and B like native vascular tissue, whereas no such stained regular lining was detected in group C. A 4-hydroxyproline assay in group C showed significantly lower levels than in groups V and B or native tissue ($P < .05$). The DNA content of the tissue-engineered vascular graft tended to be higher in group C than in groups V and B or in native tissue.

Conclusions: In the creation of tissue-engineered vascular grafts, the method of using bone marrow cells seems to be useful and superior to that of using vascular cells because bone marrow cells can be used directly, without culture.

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Received for publication Aug 6, 2003; revisions received Sept 5, 2004; accepted for publication Oct 13, 2004.

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J Thorac Cardiovasc Surg 2005;129: 1064-70

0022-5223/$30.00

doi:10.1016/j.jtcvs.2004.10.030

Many congenital heart defects are repaired by using vascular conduits such as artificial vascular grafts. However, current artificial grafts carry significant limitations, including thrombosis, infection, limited durability, and inability to grow in pediatric patients. In an attempt to overcome these shortcomings of current vascular devices, we previously reported the creation of tissue-engineered vascular graft (TEVG) constructs by using cultured autologous vascular wall cells seeded onto a synthetic biodegradable polymer scaffold.[1,2] Autologous TEVGs have the potential advantages of growth, durability, resistance to infection, and freedom from the problems of rejection, thrombogenicity, and donor scarcity. However, the use of the cultured vascular wall as a source of autologous cells for

constructing the TEVG has some disadvantages. First, the number of easily accessible vessels that can be sacrificed is limited. Second, vascular harvesting is distressing for the patient. Third, culturing of the harvested cells requires much time and expense. To overcome these disadvantages of cultured vascular wall cells, we developed the new method of using bone marrow, which can be easily obtained and used immediately, without culture. This study was designed to assess this new simple method of using bone marrow cells (BMCs) compared with the previous method of using vascular wall cells as the cell source to construct TEVGs.

Methods

Scaffold

The tissue scaffold was composed of a polyglycolic acid mesh sheet sandwiched between 2 sheets of a copolymer of polylactic acid and ϵ-caprolactone at a 50:50 ratio. The polymer matrix had more than 80% porosity with a pore diameter of 20 to 50 μm before seeding. It loses its strength in approximately 16 weeks and is degraded by hydrolysis in vivo after approximately 24 weeks (not all data are shown). These polymers were fabricated into a hybrid tubular scaffold 8 mm in diameter, 15 mm long, and 0.6 mm thick.

Cell Culture and Seeding

Venous cell group (group V; n = 4). The techniques of venous cell isolation, culture, and seeding have been described previously in detail.[2] In brief, 2- to 3-cm sections of the femoral vein were harvested from 7- to 10-kg dogs, minced, and cultured in GIT (Nihon Seiyaku Co, Tokyo, Japan) supplemented with 10% fetal bovine serum (Moregate Biotech, Bulimba QLD, Australia), 2% anti–pleuropneumonia-like organism (PPLO, penicillin G 100 U/L (Life Technologies, Gaithersburg, Md), streptomycin 100 μg/L (Life Technologies), recombinant human vascular endothelial growth factor 1.0 μg/L, recombinant human hepatocyte growth factor 0.5 μg/L, and recombinant human basic fibroblast growth factor 2.5 μg/L (Sigma Chemical Co, St Louis, Mo). The explanted tissue culture was placed in a humidified incubator maintained at 37°C with 5% CO_2 for 8 to 10 weeks. The culture medium was changed every 3 to 4 days. Approximately 6 million mixed cultured cells were seeded onto the polymer. The seeded scaffold was incubated under similar conditions for 1 week.

BMC group (group B; n = 4). Ten milliliters of BMCs was harvested from the iliac bone of 7- to 10-kg dogs. Mononuclear BMCs were separated from bone marrow with ficoil. In detail, BMCs were aspirated into a syringe containing heparin (1000 U/10 mL of bone marrow). Three milliliters of whole bone marrow was added to 3 mL of Histopaque-1077 (Sigma). After centrifugation at 400*g* for 30 minutes at room temperature, the layer of mononuclear cells was carefully aspirated. All mononuclear cells ($3.3 \pm 1.2 \times 10^6$) were seeded onto the polymer immediately. The seeded scaffold was kept in an incubator with culture media of RPMI-1640 (Sigma) supplemented with 10% fetal bovine serum (Moregate), 2% anti-PPLO, penicillin G 100 U/L (Life Technologies), streptomycin 100 μg/L (Life Technologies), recombinant human vascular endothelial growth factor 1.0 μg/L, recombinant human hepatocyte growth factor 0.5 μg/L, and recombinant human basic fibroblast growth factor 2.5 μg/L (Sigma) at 37°C with 5% CO_2 for approximately 1 hour before implantation. The total number of cells attached to the graft after 1 hour was $1.1 \pm 0.8 \times 10^6$ (Figure 1).

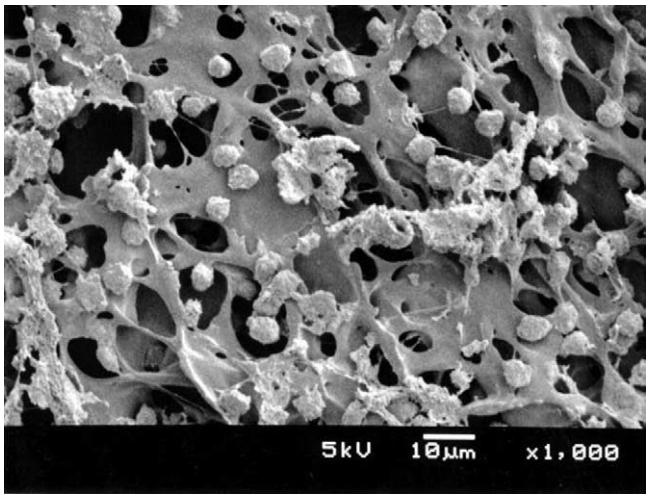

Figure 1. The electronic microscope showed bone marrow cells attached to the polymer.

Control group (group C; n = 5). After 10 mL of bone marrow was aspirated in the same method as group B to make the same basic condition, the BMCs were discarded, and no cells were seeded onto the polymer graft. This acellular polymer itself was implanted as a control graft.

TEVG Replacement

Thirteen Beagle dogs were randomized into group V (n = 4), group B (n = 4), and group C (n = 5). Each TEVG was implanted into the same dog from which the cells had previously been harvested. Anesthesia was induced with thiopental and maintained with propofol. The inferior vena cava was exposed through the sixth right intercostal space. After heparin (0.1 mL/kg) was injected, the TEVG was implanted into the inferior vena cava. The Animal Care and Use Committee of Tokyo Women's Medical University approved the use of the animals.

Evaluation of TEVG

All animals were killed and the graft was explanted at 4 weeks after implantation. The explanted graft and the adjacent native venous tissue were evaluated macroscopically and histologically.

A portion of the specimen was fixed with 4% paraformaldehyde, sectioned, and stained with hematoxylin and eosin and Masson trichrome stain for histologic examination. Additional sections of the graft were also stained with immunohistochemical techniques by using monoclonal antibodies to factor VIII (DAKO Corporation, Carpinteria, Calif),[3] anti–α-smooth muscle actin (DAKO), collagen type IV (DAKO), and CD31 (DAKO). The other portion was immersed in 0.9% saline solution and immediately processed for collagen, calcium, and DNA assays. A 4-hydroxyproline assay[4] was used to measure the collagen content in

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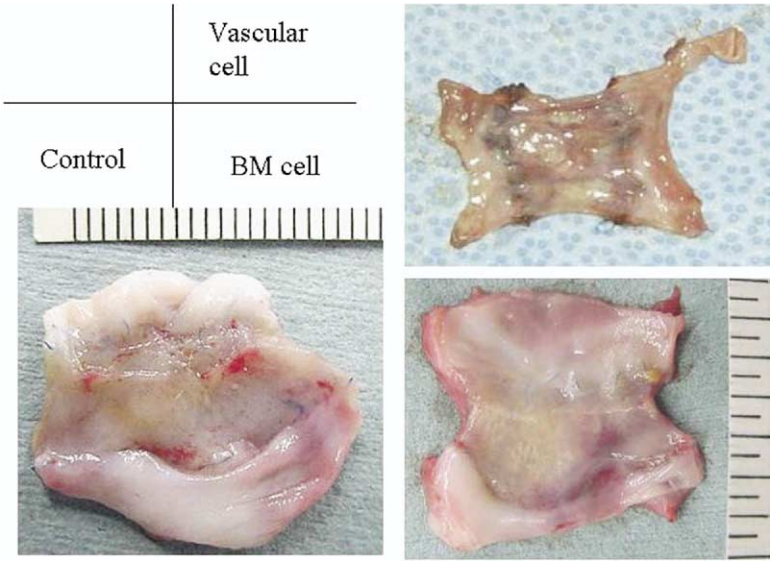

Figure 2. In groups V and B, the TEVGs showed complete endothelialization that resembled that of native vessels. In group C, incomplete endothelialization and hard tissue were observed. *BM,* Bone marrow.

the TEVG. A DNA assay[5] was used to determine the cell density in the tissue. The orthocresolphthalein complexone method was used for calcium measurement.[6]

Statistical Analysis

All results were analyzed with a statistical analysis software package (StatView version 5; SAS Institute, Cary, NC). Analysis of variance and the Bonferroni test were used to analyze the data. A probability value $<.05$ was considered statistically significant.

Results

Macroscopic Findings

In groups V and B, all TEVGs were patent and showed complete endothelialization resembling native vessels. In group C, only 2 (40%) of 5 grafts were patent. In patent grafts, incomplete endothelialization and hard tissue were observed. In all animals, the scaffold had been partially biodegraded (Figure 2).

Histologic Examination

The TEVGs were removed at 4 weeks after implantation. A regular layer of Masson stain showed evidence of collagen in groups V and B, similar to native vascular tissue. However, group C showed an irregular layer of staining that was different from native tissue (Figure 3). Immunohistochemically, groups V and B showed a layer of factor VIII–stained endothelial cells lining the vascular surfaces, whereas no such stained lining was detected in group C (Figure 4). Groups V and B also showed an anti–α-smooth muscle cell antigen immunoreactive cell lining under the endothelium, whereas no such layer was detected in group C (Figure 5). In group B, immunohistochemical staining showed CD31-positive endothelial cells and synthesis of type IV collagen under the endothelial cell layer (Figure 6).

Biochemical Examination

A 4-hydroxyproline assay showed similar levels among groups V and B and native tissue. These levels were significantly lower in group C ($P < .05$; Figure 7). The DNA content of the TEVGs tended to be a little higher in group C than in groups V or B or native tissue (Figure 8). The calcium content of these grafts was not significantly different (Figure 9).

Discussion

Various types of vascular grafts are used for the surgical treatment of cardiovascular disease. Tightly woven grafts have been used because their low porosity was thought to be preferable; however, these have the disadvantages of poor neointima formation and tissue ingrowth, which promotes calcification.[7] In low-pressure circumstances or in small-diameter vessels, these grafts also have a high risk of thrombogenesis. Cryopreserved homografts are subject to rejection and have limited long-term durability. In addition, the supply of cryopreserved homografts is greatly restricted.[8]

To address these shortcomings, efforts have been made to develop an ideal viable conduit that is nonthrombogenic, has growth potential, and will not ultimately require a replacement operation. Previously there have been reports of TEVGs used in the pulmonary artery of a lamb model[1]

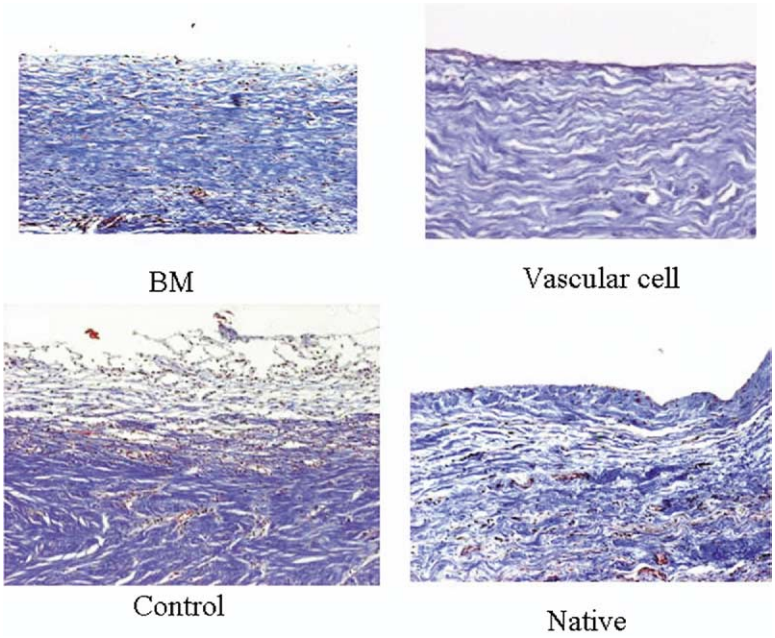

Figure 3. Masson elastic stain showed evidence of elastin and collagen in groups V and B, similar to native vascular tissue. Group C showed an irregular layer of elastic staining that was different from that seen in native tissue (original magnification, 40×). *BM,* Bone marrow.

and in the inferior vena cava of a dog model.[2] In those studies, the cells were attached to a biodegradable polymer and continued to develop structurally. Subsequently, the polymer degraded and left only the engineered tissue without any foreign material. As for the source of cells, vascular mixed cells (consisting of vascular endothelial cells, myofibroblasts, and vascular smooth muscle cells) harvested from the carotid artery or femoral vein were used. These

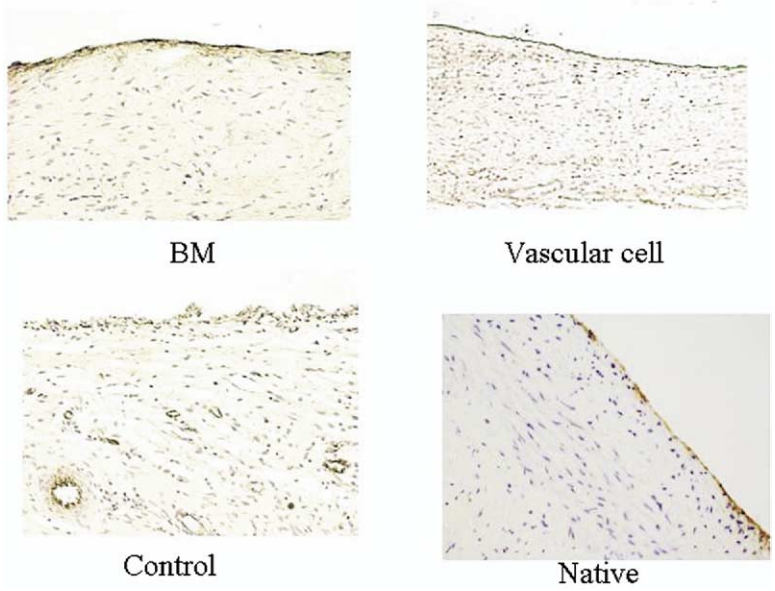

Figure 4. Groups V and B showed a layer of factor VIII–stained endothelial cells lining the vascular surfaces, whereas no such stained lining was detected in group C (original magnification, 100×). *BM,* Bone marrow.

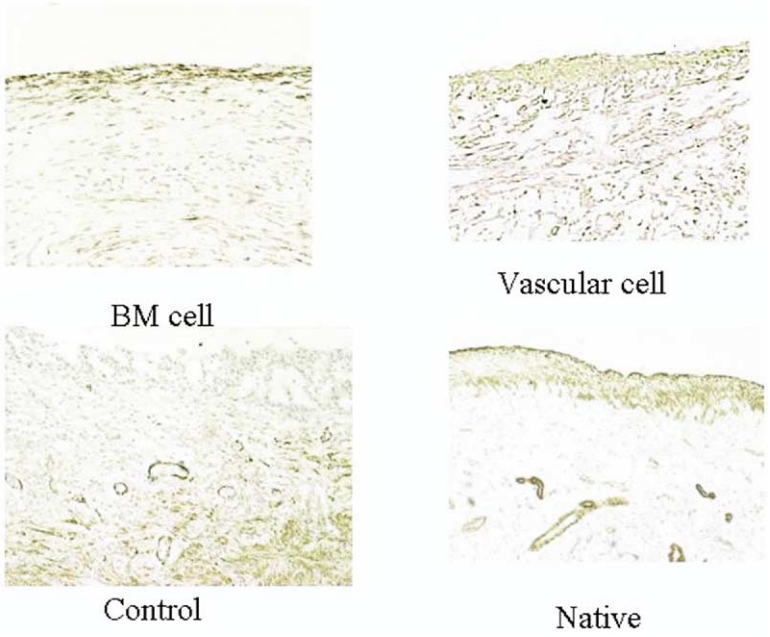

Figure 5. Groups V and B showed an anti–α-smooth muscle cell antigen immunoreactive cell lining under the endothelium, whereas no such layer was detected in group C (original magnification, 100×). *BM,* Bone marrow.

cells were cultured and seeded onto the biodegradable polymer before implantation.

However, TEVGs produced in this manner have some disadvantages. First, the number of easily accessible vessels that can be sacrificed is limited. Second, vascular harvesting is distressing for the patient. Third, cell culture after harvesting the cells requires much time and expense.

In this study, these disadvantages were overcome by the new method using BMCs—without long culture—as a source of cells for seeding the scaffolds. Recent studies have demonstrated that BMCs contain stem cells[9] that have the ability to differentiate into endothelial cells, smooth muscle cells, or fibroblasts and then to proliferate.[10] Furthermore, a prosthetic vascular graft with infiltrated BMCs showed complete endothelialization,[11] and precursors of endothelial cells with growth capacity[12] were isolated from BMCs.[13] Asahara and colleagues[14] showed that endothelial progenitor cells derived from bone marrow contributed to neovascularization in a bone marrow transplantation model.

BMCs were able to form vascular tissues similar to those of vascular mixed cells according to histologic and biochemical assays in this study. These tissues included no other structures, such as bone. In histologic studies, these tissues were similar to native vascular tissues with regard to the presence of a single layer of endothelial cells and the arrangement of collagen fiber and smooth muscle cells. In

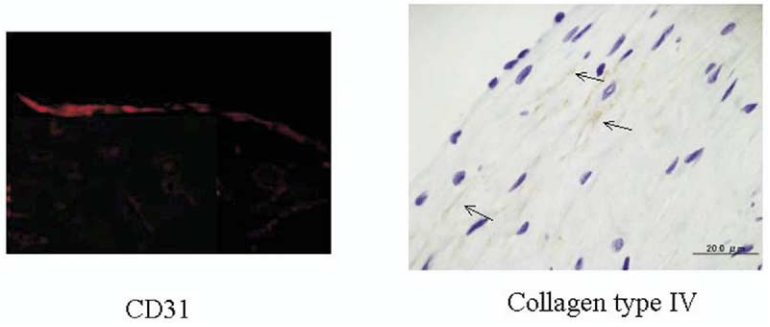

Figure 6. In group B, immunohistochemical staining showed CD31-positive endothelial cells and synthesis of type IV collagen under the endothelial cell layer.

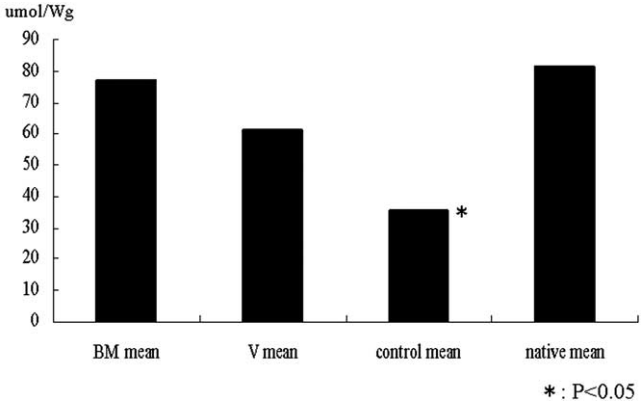

Figure 7. A 4-hydroxyproline assay showed similar levels among group V and B and native tissue. These levels were significantly lower in group C ($P < .05$). *BM,* Bone marrow; *Wg,* wet · g.

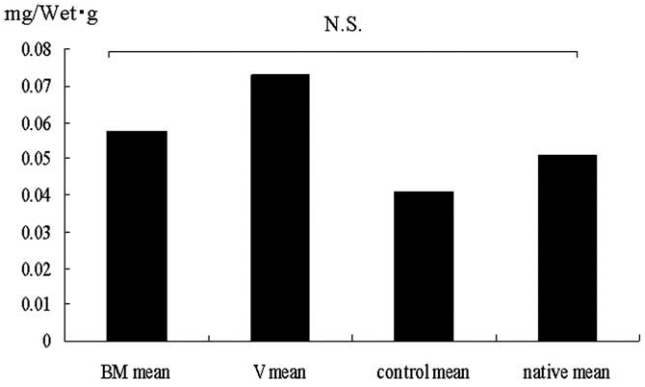

Figure 9. The calcium content of these grafts was not significantly different among groups. *BM,* Bone marrow; *N.S.,* not significant.

biochemical studies, the hydroxyproline assay showed lower levels in the control groups than in others. These results suggested that collagen formation was increased in the cell-seeding groups (groups B and V), with no relation to the type of cells, and that seeded cells on the scaffold had an important role for vascular tissue formation through unknown mechanisms. Matsumura and colleagues[15] reported that the seeded BMCs contribute to the creation of TEVG. A little higher DNA content in the control group might be attributed to the thrombosis and intimal hyperplasia in the occluded grafts. Because the follow-up term of the TEVG was short in this study, it might be difficult to detect calcification in the graft.

In a comparison of methods for creating TEVGs, the activity of seeding cells is an important factor. Because the vascular cells were harvested in a simple explanted method, there were few mixed vascular cells at first. After 4 to 6 weeks, the number of culture cells increased to 1.0×10^6 mixed vascular cells. For seeding onto the polymer, we detached these cultured cells from the flask by using trypsin. Because the trypsin has some cytotoxicity, we cultured these cells for 1 more week until their activity recovered. With regard to the multipotential stem cells in bone marrow, long culture might induce the differentiation to various lineage cells. To keep the function and activity of the stem cells, the culture period before implantation was limited to 1 hour.

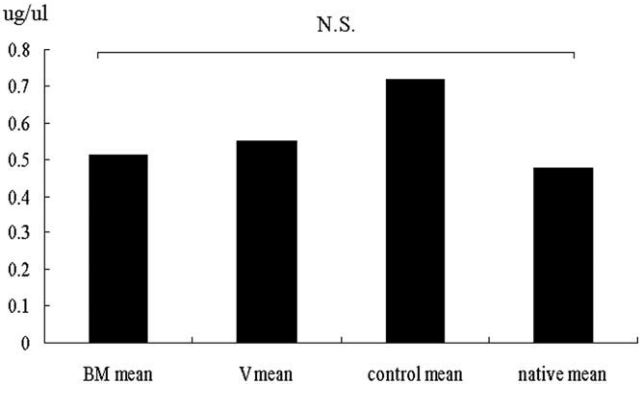

Figure 8. The DNA content of the TEVGs tended to be higher in group C than in groups V or B or native tissue. *BM,* Bone marrow; *N.S.,* not significant.

Although TEVGs have many advantages, the current TEVGs have several limitations, including high blood pressure and small diameter. Because of the weakness of the biodegradable polymer, it is difficult to use in high-pressure conditions. In small-diameter grafts (less than 3 mm in diameter), thrombosis often forms after implantation. In addition, valved conduits are more difficult with the present tissue-engineering technique because of its complex structure. To overcome these limitations, elucidation of a mechanism for creating engineered tissue might be important.

In conclusion, cell seeding onto the polymer is important in the creation of TEVGs. Our results suggest that both BMCs and vascular cells are appropriate as cell sources. With regard to the methods of creating the TEVG, BMCs may be superior to vascular cells because BMCs can be used directly, without culture.

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